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14. ABSTRACT

The focus of the work during this funding period mainly centered around Task 2, which consisted of developing standardized protocols for PCR and sequencing template preparation. The two major subtasks, development of a universal DNA extraction strategy and development of a universal PCR reaction, were initiated during the first funding period and have been completed. These accomplishments now allow us to utilize a standard DNA extraction method and a standard PCR reaction that uses the same primer set, for any unknown fungus. Confirmation of these conclusions has been obtained using a broad range of clinical specimens from both humans and animals, and demonstrates that our standard protocol works across all fungal phyla. In addition to the technical developments, we have now completed the design of the database and have been uploading sequences throughout this second year. During the course of uploading sequences, we have been testing the database since it must perform a number of tasks upon data entry. Debugging issues have been minor and although we will continue to revise the database, we now have a working version that will be used to generate output after searches.

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rDNA, SEQUENCING, PCR, BLAST

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INTRODUCTION: This proposal will develop a biocurated database of DNA sequences that can be used for the identification of human fungal pathogens. Identification of non-routine fungi from clinical specimens cannot be reliably done without specific training in mycology. Unfortunately, individuals with this training are in short supply in both civilian and military hospitals. The objective of this study is to enable laboratory technicians to make proper identifications without experience in mycology by using standardized techniques developed in this proposal to generate a DNA sequence. This sequence can then be used to search an internet-accessible database. The output from this database will result in an identification that utilizes proper and consistent nomenclature, allowing technicians to provide an appropriate identification, which will allow clinicians to more efficiently select the proper treatment course. The significance of our study will be to enable any clinical laboratory, regardless of mycological expertise, to identify any human fungal pathogen faster and more accurately than is presently possible, using a single assay.

BODY: This reporting period is the second of this award and describes work in progress on two of the four tasks (task 1 is complete, tasks 2 and 3 are in progress). Presently all tasks are on schedule and no changes to the original Statement of Work have been made. No major problems have been encountered, and because our first year was ahead of schedule, we have expanded the work on the database through more frequent meetings with our programming group. Progress on tasks 2 &3 was initiated in year one with task 2 scheduled to be completed in the middle of the second year (it has been) and task 3 scheduled to be completed in the third year. Since both tasks were started early and mentioned last year, this progress report will be briefer than the previous report. We remain on schedule and our results have been excellent. Importantly, we will include our second publication in this report (see reportable outcomes), however, the key goals of the first three tasks have allowed us to accelerate this project and have resulted in an additional 10 manuscripts that have been submitted or are in preparation.

Progress report on Task 1. **CREATION OF AN INTERNET-ACCESSIBLE, SEQUENCE DATABASE FOR MOLECULAR IDENTIFICATION OF ALL KNOWN HUMAN FUNGAL PATHOGENS.** (Months 1-12). This task was reported as complete in the first annual progress report. For questions, please refer to the first report, or we can resend a copy of the file if needed.

Progress report on Task 2. **DEVELOPMENT OF STANDARDIZED PROTOCOLS FOR PCR (POLYMERASE CHAIN REACTION) AND SEQUENCING TEMPLATE PREPARATION.** (Months 6-18)

This task was largely completed in the first year. Additional data generated in the second year essentially finishes this task and confirms that we have a standardized method for preparing template DNA from any unknown specimen that can then be successfully amplified with our universal primers. Table 1 shows additional data generated from the same experiment as reported during the last report period. The results are unchanged even with a broader spectrum of fungi and reveals that our protocol amplifies any unknown yeast, and ~91% of unknown molds, for a combined success of 95%. Isolates represented all phyla of fungi and various types of isolates including human, animal, plant, soil, and water.

Table #1. DNA isolation success rate.

Morphology	No. Preps	Succeeded	Failed	% Successful
Yeast	162	162	0	100
Hyphal	267	238	29	90.8[1]
Total	429	400	29	95.45

[1]This rate can be increased by another 6% upon repeat attempts or different culture conditions that are not our standard prep. After these modifications, we found only ~3-4% cannot be amplified using our simplified approach. When longer preps are employed, these remaining isolates were all amplified.

The major advantage of our strategy is that, as planned, our DNA preparation method requires no hazardous agents, such as phenol, and does not require supernatant transfers to new tubes, thereby lessening PCR contamination potential. Additionally, our universal primer results and PCR strategy have just been recently applied by collaborators, with success, to unknown samples obtained from military burn patients (see reportable outcome #4). These results, shown in Fig 1, demonstrate the robustness of our approach, as the samples were formalin-fixed, paraffin embedded sections.

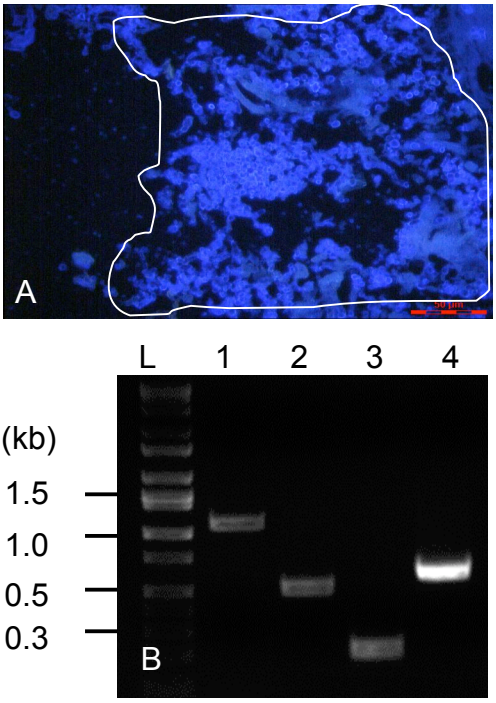

Fig 1. PCR amplification of fungal isolates from fixed specimens. A) Laser capture microdissection (white border) was used to recover fungal elements from paraffin sections from a burn patient. B) After DNA preps, the samples were amplified with combinations of primers used in our study. All primer combinations yielded a PCR product. Lanes 1-ITS1+NL4, 2-ITS1+ITS4, 3-ITS1+ITS2, 4-NL1+NL4. Primer combination ITS1+NL4 is our universal primer pair. The identification of this fungus was *Candida tropicalis*.

Since task 2 is complete, we now will use our standardized preparation and primer combination for all isolates. Improvements will be investigated as opportunities arise, and presently are focused on two areas. The first is the use of Whatman FTA™ cards, which are normally used for blood typing and were investigated in the first year. These cards may be useful for shipping templates through the mail. They are employed, with subtle variations, just as for blood specimens where a sample is spotted on a card, allowed to dry, washed, and then a small punch is taken and used directly (by dropping the small punch-out into a microfuge tube) as a PCR template. The utility of these cards is ease of shipping, multiple sample preps from one specimen, and also as an archiving method. Preliminary studies were done in the first year with encouraging results, and have now been extended to additional molds. We will continue these

studies and develop a protocol that utilizes the cards as a back up plan to our standardized approach. The advantage of using this strategy is that field hospitals or forward areas with limited laboratory equipment might be able to use this method in place of sending live cultures back to rear areas. Additionally, the cards have already been shown to be able to release DNA from fungi that can be PCR amplified (1). A second area that we will investigate is an automated DNA preparation method. Presently, our method uses a single tube approach, which works fine. There are a number of magnetic bead extraction methods that are on the market, which can be scaled up for high throughput, and also have been applied to fungi (2). These methods use proprietary lysis solutions, which may or may not fit with our strategy. The magnetic bead technique, however, could be useful if analyses are performed in centralized areas. Importantly, our investigation of this method will incorporate our rapid lysis protocol in place of the protocol that comes with the beads. Although no biological system will perform at a level of 100% efficiency, our reasoning for investigating this approach is that with instrumentation, the magnetic bead method is scalable for high throughput, and is already used in many clinical laboratories for processing blood and other body fluid specimens. We also are interested in seeing if we can get even closer to a 100% success for rapid DNA isolation from molds, which we currently recover at 91% success rate for first time preparations. Even with repeat attempts, ~3-4% need our more complicated prep methods (3), which have all been successful, but are not the standard approach.

Progress report on Task 3. **GENERATION OF TYPE SEQUENCES**. (Months 6-36)

The generation of type culture sequences is on schedule and was initiated as soon as we had a workable standardized DNA template preparation protocol. These cultures have been obtained from our collection, and the United States Department of Agriculture (USDA) at no charge, as well as being purchased from the American Type Culture Collection (ATCC) (USA) and the Centraalbureau voor Schimmelcultures (CBS) (Holland). The purchased cultures, except for BSL3 pathogens, have been obtained from CBS because they are much cheaper than the ATCC cultures. This process requires substantial import documentation, however, since we have done this multiple times, we generally can obtain the cultures in a timely fashion and have gone through the process 3-4 times. To save funds on the shipping, we generally purchase sets of 100-200 isolates, and will probably only need to make one more major purchase. Template preparation using our methodology has proceeded without any problems, in spite of the diverse variety of fungi that we are collecting. In fact, the only isolates that have not worked (documented in Table 1) with the standard method are clinical isolates obtained as redundant (non type) cultures from our own institution. All the type cultures can be prepared using our standard methods.

The sequences obtained from each culture are placed on our desktop database and from there, are uploaded onto the website database. No problems have been encountered and we anticipate that this process will continue without any major difficulty. Both the live cultures and the sequences obtained from them are backed up in multiple ways. The live cultures are stored frozen in three separate locations as sub cultures and the sequences are stored in three separate locations, including back up drives. In summary, this task is largely a data-gathering task in

which we obtain and bank as much data as we can. In addition to obtaining and storing data, we are banking our culture collections, as well as the DNA preparations that come from them. These preparations will be saved in case additional sequencing is required.

KEY RESEARCH ACCOMPLISHMENTS:

- Our basic standardized template preparation, PCR (and sequencing) approach, has proven robust enough for other applications, in particular, fixed specimens.

- Since we have been saving our DNA preps, we have made them available to investigators interested in obtaining sequences for other genes. At our working group meeting, a group from the CDC has expressed interest in a collaboration using these reagents (see below).

- A second research collaboration that uses our expertise in addition to the funded NIH proposal, has been initiated (see below)

REPORTABLE OUTCOMES:

In addition to one of the previously reportable outcomes that continued through this year (Last year's item #4, Co-investigator on a funded National Institutes of Health proposal entitled "Detection and significance of antifungal resistance in oropharyngeal candidiasis". Awarded 07/06 and runs until 06/11), the following outcomes are noted;

1) A second publication is reported this period: Drees M, Wickes BL, Gupta M, Hadley S. *Lecythophora mutabilis* prosthetic valve endocarditis in a diabetic patient. Med Mycol. (2007) 45(5):463-7.

2) Ten additional manuscripts have been submitted or are in preparation. This productivity is the result of the completion of task 2, which yielded a standardized method that we have used for fungus identification in multiple collaborative studies. The manuscripts all deal with identification of infecting fungi from a diverse variety of sources, and demonstrate the robustness of our strategy. The manuscripts should be in press or published by the next reporting period.

3) I was invited to participate in a Center for Disease Control (CDC) – sponsored session on DNA sequence-based species identification during the 18th Annual Focus on Fungal Infections Meeting held in San Antonio, TX. I served as a discussion moderator. The function of this meeting was to establish a CDC-organized working group on the molecular identification of fungi, which I am now a part of. Preliminary plans are for this group to meet annually.

4) A proposal was submitted to the Infectious Disease Clinical Research Program, National Institutes of Allergy and Infectious Diseases, entitled, *Laser microdissection (LMD) with DNA PCR: A novel method for determining the etiology of fungal burn wound infection* with (Principle Investigator) Laurie Davignon, MD, Maj, USAF, MC, Assistant Chief, Infectious Disease Service, San Antonio Military Medical Center, Fort Sam Houston, TX 78234-6200, and other

collaborators. Our role as a collaborator will be to provide the fungal identifications using the system developed in this proposal.

5) Anna Romanelli (Graduate student supported by the award) presented a seminar entitled, *Development of a Biocurated Sequence Identification Database for Molecular Identification of Human Fungal Pathogens* at the 2008 annual South Central Medical Mycology Meeting, Oct 26-27, San Antonio, TX.

CONCLUSION: In this second reporting period, we have continued work on the three tasks started in the first year. Task 1 is now complete as we have finalized a standardized protocol for preparing template DNA and used this protocol to obtain sequencing data. The standardization of our methodology has allowed us to generate type culture data (the key data in our database), which we have done continuously and will extend into the next reporting period. No problems have been encountered as we gather this data. In addition to developing the methodology for this study, we have successfully applied this methodology to other studies as one proposal has been funded in which we are collaborators, and a second proposal has been submitted, in which we will also collaborate on. Finally, I have been invited to participate in a working group sponsored by the CDC. During our recent meeting of about 20 scientists from around the world, our immediate goal will be to develop guidelines for molecular identification of fungi, which will be published in a suitable journal. We anticipate that many of our approaches will be incorporated into these guidelines.

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APPENDICES:

1) Publication (from year 1): Bar-Meir M , Sutton DA , Wickes B , Kurtzman CP , Goldman S , Zheng X. 2006. Catheter-related fungemia due to *Candida thermophila*. J Clin Microbiol. 44:3035-6.

2) Publication (from year 2): Drees M, Wickes BL, Gupta M, Hadley S. 2007. *Lecythophora mutabilis* prosthetic valve endocarditis in a diabetic patient. Med Mycol. 45:463-467.

SUPPORTING DATA: N/A

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Catheter-Related Fungemia Due to *Candida thermophila*

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We report a case of bloodstream infection caused by *Candida thermophila*, a yeast not previously associated with human disease. The infection occurred in a 13-year-old boy with medulloblastoma who presented with 1 day of fever. Multiple blood cultures were positive for yeast. Removal of the catheter resulted in prompt resolution of the fever and sterilization of the blood cultures. The species was identified by sequencing domains 1 and 2 of the large subunit rRNA gene. Antifungal susceptibility testing was also performed.

CASE REPORT

A 13-year-old boy with medulloblastoma presented to the emergency department because of a 1-day history of fever up to 39.3°C, decreased oral intake, and increased fatigue. The tumor was diagnosed 14 months prior to his presentation. The patient was treated according to the Children's Oncology Group A-9961 protocol with surgical resection followed by reduced-dose craniospinal irradiation and alternate cycles of cisplatin, vincristine, and cyclophosphamide. The last cycle was given 3 weeks prior to his presentation. A central venous catheter, in place for a year, was used for administration of chemotherapy and hyperalimentation. The patient also received *Pneumocystis jiroveci* pneumonia prophylaxis with trimethoprim-sulfamethoxazole (160 mg of the trimethoprim component twice daily for three consecutive days each week).

Physical examination showed a febrile but otherwise well-appearing boy. The central line site showed no signs of infection or inflammation. Total white blood cell count was 4,300/mm^3 with 3,400 neutrophils/mm^3 and 560 band forms/mm^3, hemoglobin was 8.1 g/dl, and platelets were 59,000/mm^3. Findings on a chest radiograph were normal. A blood culture was drawn from the central line; the patient was given a dose of ceftriaxone and was sent home. The blood culture grew yeast after 24 h, and the patient was called and admitted to the hospital. At that time he was still well appearing and afebrile. An additional set of central and peripheral blood cultures was obtained, and administration of intravenous liposomal amphotericin (AmBisome) at 200 mg (5 mg/kg of body weight) once a day was begun. Altogether, eight sets of standard blood cultures (BACTEC Peds plus/F and standard anaerobic/F for each) and four sets of fungal blood cultures (ISOLATOR 1.5; Wampole Laboratories) were drawn over a 5-day period, and nine (five of the standard blood culture bottles and all fungal cultures) grew yeast. Sterilization of the blood was achieved only following removal of the central venous catheter on the fifth day of the antifungal therapy. The patient completed 6 weeks of liposomal amphotericin therapy and recovered without complications.

Laboratory findings. The yeast isolate from the patient grew after 24 to 48 h of incubation at 37°C. The colonies were moist and white in color. The germ tube test was negative. No hyphae or pseudohyphae were observed. The isolate was evaluated by the Microscan Walkaway system with a yeast identification plate (Dade Behring) and the API 20C AUX system (bioMerieux). Both gave an identification of *Hansenula polymorpha*. When biochemical reactions were run independently of the rapid systems, the isolate was negative for urease and positive for nitrate and glucose. The yeast grew at 37 and 42°C but not at 50°C.

Since the yeast could not be identified satisfactorily with the Microscan and API identification systems, DNA sequencing was conducted to provide identification. The isolate was identified as *Candida thermophila* (9) from its unique DNA sequence in domains 1 and 2 (D1/D2) of the large subunit rRNA gene by the National Center for Agricultural Utilization Research in Peoria and by the University of Texas Health Sciences Center in San Antonio. As described earlier (5, 6), genomic DNA was extracted from the yeast cells and combined with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) in a PCR. The resulting D1/D2 amplicon of ca. 600 nucleotides in length was purified, and both DNA strands were sequenced using primers NL-1 and NL-4 and an ABI (Applied Biosystems) automated DNA sequencer. The GenBank accession number for this sequence is DQ402185. The sequence of the isolate differed from that of *Candida thermophila* (GenBank accession AF283568) by one nucleotide. Other phylogenetically closely related organisms included *Pichia salicis* (GenBank accession AF403148; 99% identity), a presently undescribed species, and *Pichia angusta* (GenBank accession U75524; 98% identity) (4, 6). Our isolate has been deposited with the ARS Culture Collection as NRRL Y-27863 and with the American Type Culture Collection (ATCC MYA-3665).

Antifungal drug susceptibility testing was performed by the broth microdilution method based on the CLSI (formerly NCCLS) guidelines (8). Briefly, RPMI medium was used. The inoculation size was 5×10^4 CFU. MICs were read

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TABLE 1. MIC and minimum fungicidal concentration results

Drug	MIC (μg/ml) at 24 h	MIC (μg/ml) at 48 h	MFC[a] (μg/ml)
Amphotericin	≤0.03	0.125	0.25
Caspofungin	0.12	0.5	1
Flucytosine	0.12	2	≥128
Fluconazole	1	2	16
Itraconazole	0.06	0.25	0.5
Ketoconazole	≤0.03	0.125	1
Voriconazole	≤0.03	≤0.03	≤0.03

[a] MFC, minimum fungicidal concentration.

at 24 and 48 h by comparing the turbidity of test wells to that of the untreated controls. A change in turbidity equal to or greater than 90% compared to drug-free control results was used to establish MIC breakpoints. The minimal fungicidal concentration results were obtained by recording colony counts on plates. Results are summarized in Table 1.

Discussion. Invasive candidiasis is an important cause of morbidity and mortality in chronically or critically ill patients (2, 3). Infections caused by *Candida* species are the fourth most common cause of nosocomial bloodstream infection in the United States (1, 11), with species other than *Candida albicans* emerging as pathogens. The non-*C. albicans* yeasts are often associated with resistance to antifungal azoles and with higher mortality. We describe the first reported case of *Candida thermophila* causing a human infection.

C. thermophila was described as a thermophilic soil yeast capable of growth at 50°C (9). Although the current isolate did not grow at 50°C, it did grow well at 37 and 42°C. Since it is difficult to identify this species with either commercial or conventional biochemical assays, this characteristic of growth in elevated temperature can be an indication for further analysis such as rRNA gene sequencing. Identification of yeasts from the large subunit rRNA gene D1/D2 sequence comparisons has been highly reliable. Strains of the same species ordinarily show only 0 to 3 nucleotide differences (6), but a few exceptions to this pattern have been found. For example, *Candida guilliermondii* and *Candida fermentati* differ by 3 nucleotides in large-subunit D1/D2 but show only 40% relatedness when compared by nuclear DNA reassociation (10). Consequently, these two taxa are closely related but not conspecific. The current isolate was identified as *C. thermophila* based on its close genetic similarity to the type strain of this species. The single nucleotide difference with the type strain has been interpreted as intraspecies strain variation. Gene sequence analysis has been successfully used for the identification of pathogenic fungi in addition to analysis of morphological and biochemical characteristics (7).

Although this is the first reported case of *C. thermophila* causing candidemia in a human, this may not be the first case of invasive disease due to this recently identified yeast, because identification and differentiation of yeasts on the basis of morphological and biochemical characteristics can be difficult. Therefore, the incidence and prevalence of this organism and its pathogenic role might be underestimated.

Many *Candida* species causing invasive infections have been non-*C. albicans* yeasts such as *C. krusei* and *C. glabrata*. These species can be inherently (primarily) or secondarily resistant to fluconazole and may be more difficult to treat. The isolate from our patient was susceptible to all antifungals in vitro, and the patient was treated successfully with liposomal amphotericin, although fluconazole might have been as effective.

In summary, as the population of immunocompromised hosts grows, organisms previously not considered as pathogens might cause invasive disease. *C. thermophila* should be added to the long list of yeasts that can cause bloodstream infections in the immunocompromised or critically ill patient.

Nucelotide sequence accession number. The sequence of the D1/D2 amplicon described in this study has been deposited under GenBank accession no. DQ402185.

We thank Annette W. Fothergill, Dora I. McCarthy, Mollyn Valentin, and William Kabat for technical assistance. We also thank Stanford Shulman for helpful discussion and review of the manuscript.

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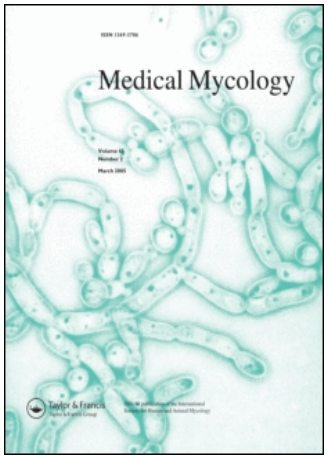

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Lecythophora mutabilis prosthetic valve endocarditis in a diabetic patient

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Case Report

Lecythophora mutabilis prosthetic valve endocarditis in a diabetic patient

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While dematiaceous (dark-walled) fungi are ubiquitous in the environment, their involvement in invasive human infections has rarely been reported. However, these organisms have been identified as potential emerging pathogens, particularly among immunocompromised hosts. We describe a diabetic patient with *Lecythophora mutabilis* prosthetic valve endocarditis who was treated surgically, as well as with amphotericin B lipid complex and voriconazole, which were subsequently followed by prolonged voriconazole suppressive therapy. To the best of our knowledge, our patient is the first reported survivor of *L. mutabilis* prosthetic valve endocarditis.

Keywords *Lecythophora mutabilis*, endocarditis, dematiaceous fungi, phaeohyphomycosis

Introduction

The dematiaceous fungi, e.g., *Lecythophora mutabilis*, are characterized by the presence of melanin or melanin-like pigments in the cell walls of their hyphae, conidia, or both. Melanin is considered a virulence factor due to its antioxidant and other properties [1,2]. With at least 109 species from 60 genera [3], these saprophytic fungi are widely distributed in the environment and are found in soil, wood, vegetative matter, and polluted water. These fungi have undergone considerable reclassification over time. Obsolete synonyms for *Lecythophora mutabilis*, include *Phialophora mutabilis* and *Margarinomyces mutabilis*. Dematiaceous fungi have long been recognized as causative agents of mycetoma and chromoblastomycosis, but cases of invasive disease (disseminated phaeohyphomycosis) appear to be increasing, as these organisms take advantage of the biologic niche provided by increasing numbers of immunocompromised patients [1].

Case report

A 58-year-old man with coronary artery disease, diabetes, chronic obstructive pulmonary disease (COPD), congestive heart failure (CHF), chronic renal insufficiency and critical aortic stenosis underwent bypass grafting and aortic valve replacement with a porcine tissue valve in July 2004. In October 2004, an automatic cardiac defibrillator was implanted, but the surgery was complicated 10 days later by a methicillin-resistant *Staphylococcus aureus* (MRSA) pocket infection and bacteremia. The device was explanted and he completed a 6-week course of appropriate antibiotics. Between November 2004 and February 2005, the patient was hospitalized multiple times at another facility for fever and shortness of breath, attributed to COPD exacerbations and treated with antibiotics and steroids. Blood, urine, and pleural fluid cultures

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remained negative. To evaluate persistent fever, in February 2005 a transesophageal echocardiogram (TEE) and tagged white blood cell scan were performed, both of which were negative. One month later, he was afebrile but was noted to have a leukocyte count of 16.4×10^3 cells/μl, with 76% neutrophils and 6% eosinophils, and an elevated erythrocyte sedimentation rate and C-reactive protein. Blood cultures obtained at this time remained negative.

Approximately three weeks later, the patient presented again to the same facility with fever, chills, pleuritic chest pain. A TEE failed to reveal the presence of vegetation. He developed electrocardiographic changes and worsening CHF and was transferred to our facility. A repeat TEE revealed a large echodensity encasing and restricting the aortic valve leaflets and obstructing the aortic outflow tract. The patient was taken for emergency valve replacement, and was found to have a 4–5 cm lobulated dense mass originating from the entire aortic valve prosthesis (Fig. 1). Tissue Gram stain revealed 3+ neutrophils and 2+ fungal elements. All blood cultures obtained at both facilities remained negative.

Histologic examination of the prosthetic aortic valve revealed innumerable non-pigmented, septate, hyphal fungal elements (Fig. 2) with bulbous swelling and branching reminiscent of *Aspergillus* or *Pseudallescheria* species. The valve was cultured on blood, chocolate and EMB agars without any growth. However, low, waxy colonies with feet and a few white aerial hyphae were evident within 48 h of inoculation on brain heart infusion (BHI) and Sabouraud dextrose agar (SAB) in tubes and SAB in plates. The colonies matured within five days, becoming salmon-colored

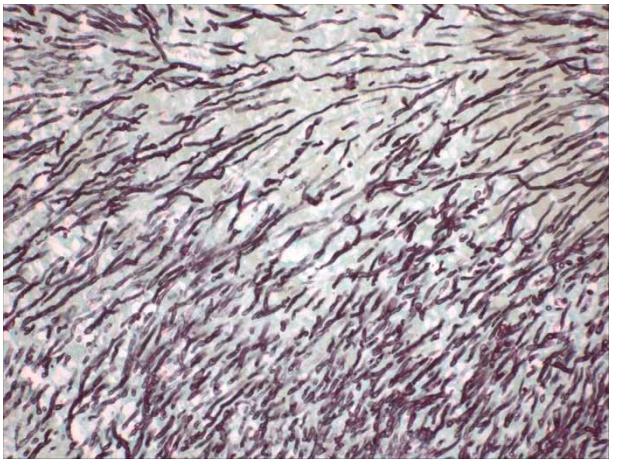

Fig. 2 Gomori methenamine silver (GMS) stain of aortic valve, showing innumerable fungal elements due to *Lecythophora mutabilis*. (See color online.)

and acquiring a central brown pigmentation (Fig. 3). Lactophenol cotton blue (LCB) scotch tape preparation showed development of long thin, hooked conidia, some of which showed swelling with long thin extensions, rounded ends and large vacuoles.

Biochemical testing did not identify the organism, and samples were sent to the Fungus Testing Laboratory at the University of Texas Health Sciences Center (UTHSCSA) for further identification. At UTHSCSA it was provisionally identified as *Lecythophora mutabilis* based on its morphology on potato flakes agar, with cream to yellowish colonies that darkened with a yellow-orange periphery and production of dark chlamydoconidia [4]. Confirmatory DNA sequencing of the D1/D2 region (large subunit RNA gene) was performed at the UTHSCSA Advanced Nucleic Acids

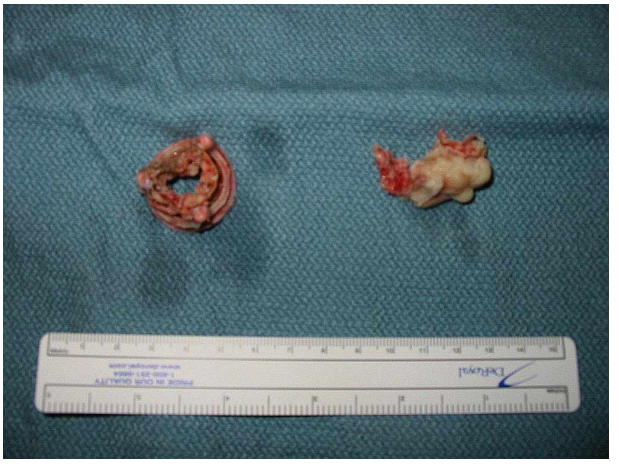

Fig. 1 Prosthetic aortic valve (left) and large, obstructing vegetation (right). (See color online.)

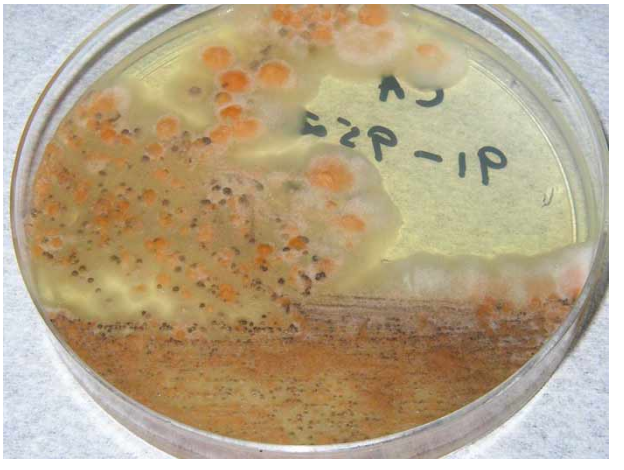

Fig. 3 Appearance of mature colonies *Lecythophora mutabilis* on Sabouraud's dextrose agar. (See color online.)

Core facility. A BLASTn search of the NCBI database found a match of 575/575 bases (100% identity) for *L. mutabilis*. The sequence was deposited in Genbank under accession number EF517490. Fungal susceptibility testing was performed in accord with the Clinical Laboratory Standards Institute M-38-A broth microdilution method [4], and minimum inhibitory concentrations (MIC) at 48 h revealed susceptibility to amphotericin B (MIC 0.25 μg/ml), caspofungin (MIC 2 μg/ml), voriconazole (MIC 0.125 μg/ml), and posaconazole (0.03 μg/ml). The isolate was sent for deposit to the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Alberta, Canada, where it was accessioned as UAMH 10554.

Postoperatively, while fungal identification and susceptibilities were pending, the patient was treated with amphotericin B lipid complex (ABLC) and oral voriconazole. Dose adjustment was required for worsening renal function while on ABLC. He remained afebrile and was discharged home on postoperative day 32. After a 6-week course, ABLC was discontinued due to renal toxicity and the availability of antifungal sensitivities, and the patient was continued on oral voriconazole. Subsequently, his recovery was complicated by an MRSA catheter-related bloodstream infection, followed by recurrent endocarditis and perivalvular abscess. Blood cultures revealed MRSA only and he was not deemed a candidate for further surgery, limiting our ability to rule out persistent *L. mutabilis* infection. No embolic or metastatic infections occurred during either episode of endocarditis. After prolonged intravenous anti-staphylococcal and antifungal treatment, he remains well, as of 23 months postoperatively, on chronic suppressive therapy with minocycline and voriconazole.

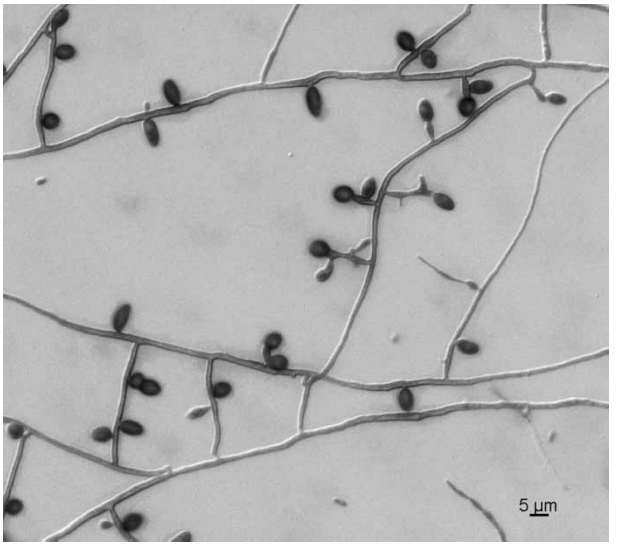

Fig. 4 Pigmented chlamydospores characteristic of *Lecythophora mutabilis*. Published with permission by Lynne Sigler, MSc, Curator, University of Alberta Microfungus Collection and Herbarium.

Discussion

Endocarditis and other invasive infections due to dematiaceous fungi have been reported in a variety of hosts, including premature neonates [5], diabetics [6], bone marrow transplant recipients [7], hemodialysis patients [8], and solid organ transplant recipients [9]. A review [1] of 72 reported cases of disseminated phaeohyphomycosis revealed heart valve infections in 21 (29%) and overall mortality of 79%. In a retrospective review [10], 152 reported cases of fungal endocarditis cases were identified during 1995–2000, of which 39 (26%) were due to molds. Six (15%) of the latter cases were due to dematiaceous fungi. Notably, the mortality rate for mold-related endocarditis was significantly higher than that due to yeast (82% vs. 40%).

L. mutabilis has been described as a cause of invasive disease in humans, including endophthalmitis [11,12] and relapsing fungal peritonitis in a peritoneal dialysis patient [13]. Endocarditis due to *L. mutabilis* has been described only twice previously, both in the 1970s and involving prosthetic valves. The first case was a 56-year-old woman with a porcine mitral valve [14], and the second a 47-year-old man with mechanical mitral and aortic valves [15]. Neither patient was known to be immunocompromised and, similar to our patient, both had negative blood cultures and large, obstructing vegetations. Interestingly, all three patients were noted to have peripheral eosinophilia (which was transient in our patient's case), an uncommon finding in fungal infections. It has been suggested that phaeohyphomycosis be considered in the differential diagnosis of eosinophilia [1]. Seeing as both of these previous cases were fatal, our patient, to the best of our knowledge, is the first reported survivor.

The source of our patient's infection remains unclear. Possibilities include environmental contamination at the time of surgery, contamination of the valve at the site of manufacture, or environmental exposure of the patient after surgery. No additional cases of *L. mutabilis* have been reported from our institution. The patient used well water at home; samples were obtained but *L. mutabilis* was not isolated. No other environmental risk factors were identified.

Treatment options for invasive infections with dematiaceous fungi can be quite limited. While our patient's *L. mutabilis* isolate appeared quite sensitive to a variety of antifungal agents, many related fungi are resistant to amphotericin B, often considered the ideal empiric and definitive treatment for fungal infections [1]. Voriconazole has been used successfully to treat *L. mutabilis* endophthalmitis [12] and infections caused by other dematiaceous fungi [16–19], but treatment failures have also been reported [20–22]. We elected to treat our patient with both ABLC and voriconazole because of the severity of his infection and delay in acquiring antifungal sensitivities. However, to date there has been limited data supporting the use of multiple antifungal agents. In addition, the relationship between *in vitro* antifungal activity and clinical efficacy remains unclear. When possible, surgical as well as medical management is likely required. Because of the recurrent episode of endocarditis, during which *L. mutabilis* could not be definitively ruled out, we elected to maintain our patient on chronic suppressive oral voriconazole. This has now continued for nearly 2 years, with no evidence of recurrence and no adverse effects thus far.

The dematiaceous fungi, including *L. mutabilis*, are capable of causing life-threatening, invasive infections even in patients who are not traditionally considered highly immunocompromised. Our patient's risk factors for fungal disease included significant antibiotic exposure and intermittent steroid use, but diabetes was his only active immunocompromising condition. Clinicians should remain aware of these emerging pathogens, as their diagnosis may be difficult to ascertain using traditional methods. Blood cultures frequently remain negative and, for endocarditis, repeated echocardiography may be required. Intensive and prolonged microbiological effort is required to identify these fungal pathogens. Broad antifungal therapy and early surgical management likely contributed to our patient's survival.

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Potential conflicts of interest

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